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The Genetic Regulation of Human Sleep-Wake Rhythms and Patterns

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I INTRODUCTION

We are creatures of habit. This is evident in our molecularly derived need for sleep. Beneath those molecules are a highly conserved set of genes that encode for the adaptation to our planet's 24 h solar cycle. Environmental signals like light, social interactions, and food provide the cues necessary for the regular organization of cellular function, culminating in efficient organismal behavior. Sleep is a required behavior for most organisms (Chong, Xin, Ptacek, & Fu, 2015). The conceptual framework for sleep proposes that sleep need and the timing of sleep are integrated processes. Sleep therefore can be divided into processes controlling the timing of sleep (Process C) and the drive for sleep (Process S) (Borbély & Wirz-Justice, 1982; Fisher, Foster, & Peirson, 2013). In humans, under normal conditions, a circadian rhythm (which is believed to drive Process C) "primes" the body to consolidate the timing of sleep to epochs of time when the functions of sleep are most efficient. The sleep homeostat (believed to drive Process S) regulates the duration of sleep so that "sleep need" is met and appropriate vigilance, cognition, and motor functions can be restored for the following wake period. Thus, these two processes dictate when and how we sleep.

The biological consequences of sleep and circadian disruption are becoming more apparent, yet societal demands on personal schedules and sleep patterns remain high. Epidemiological data in American workers point to the steady reduction in sleep length over the past 50 years (Kraus & Rabin, 2012), with fluctuations in sleep duration between the workweek and weekend (Roenneberg, Allebrandt,

Merrow, & Vetter, 2012). Sleep deprivation, social jet lag, chronic sleep restriction, and circadian desynchrony have been associated with a variety of medical comorbidities, including cancer (Haus & Smolensky, 2013), metabolic and immune dysfunction (Eckel-Mahan & Sassone-Corsi, 2013; Prather, Janicki-Deverts, Hall, & Cohen, 2015; Roenneberg et al., 2012), and cognitive and psychiatric alterations (Jagannath, Peirson, & Foster, 2013; Leung et al., 2015). Clearly, the incidence of sleep disruption is significant and likely will continue to increase. Thus, the comorbid consequences on human health could be enormous on a population scale. It is estimated that 50–70 million individuals suffer from chronic sleep disorders in the United States alone (Colten & Altevogt, 2006). A call for further investigations of sleep and circadian rhythm regulation has implications for not only novel treatment of sleep disorders but also the improvement of the efficacy and safety of existing therapies if they can be administered in the context of an appropriate, individualized biological phase (chronotherapy) (Hermida et al., 2013).

A better understanding of the molecular pathways governing the timing and duration of sleep leads to increased clarity of the roles of sleep and circadian disruption on these processes. The fundamental machinery underlying circadian rhythms has largely been elucidated in model systems such as *Drosophila* and mouse (Mohawk, Green, & Takahashi, 2012; Rosbash, Allada, Dembinska, Guo, & Marrus, 1996). Each cell in the body has a molecular clock that governs the expression of clock-controlled genes (a percentage of the genome), which varies depending on the cellular subtype (Bozek et al., 2009; Keller et al., 2009), to provide a periodicity

(the time required for one complete oscillation to occur) or phase (e.g., time of awakening relative to start of the solar day).

The clock itself can be described as a series of highly coordinated, autoregulatory feedback loops. The core heterodimer of the clock is made up of the proteins CLOCK and BMAL1, which activate transcription of genes that have clock gene promoters (E-box elements). CLOCK/BMAL1 drives the transcription of negative and stabilizing regulatory loops of the clock itself, including three period genes (*PER1*, *PER2*, and *PER3*) and two cryptochrome genes (*CRY1* and *CRY2*) that represent a cohort of negative regulatory proteins. PER and CRY are posttranslationally modified and heterodimerize and enter the nucleus where they inhibit CLOCK-/BMAL1-mediated transcriptional activity. This process occurs reliably in a roughly 24 h period that can be reset by external cues (i.e., peripheral mediators from the SCN or, in the case of the SCN itself, light) and provides a cell-autonomous timekeeping process. Many other proteins are under clock control that affect clock stability and function. TIMELESS functions in the mammalian clock by binding PER and CRY and has independent transcriptional repressor activity of core clock machinery (Gottter, 2006). CLOCK/BMAL1 also mediates transcription of Rev-ERBa and RORa that inhibit and activate the transcription of *BMAL1*, respectively. Similarly, E4BP4 negatively regulates and DBP positively regulates D-box elements in clock genes, which contributes to rhythmic transcription of RORa among other components of the clock. Other components of the circadian clock regulatory machinery have been identified, such as DEC1 and DEC2, which repress the transcriptional activity of CLOCK/BMAL1 at promoter sites.

Adding another layer of regulation, many of these proteins are posttranslationally modified to affect stability and speed of the clock. Casein kinase 1 epsilon and delta (CK1 ϵ and CK1 δ) regulate the negative feedback loop of the clock by phosphorylating specific residues on PER proteins. Depending on the site of phosphorylation, protein translocation to the nucleus can be promoted, or other sites can prioritize protein turnover. Additionally, posttranslational regulation by O-GlcNAcylation (an event mediated partially by glucose levels) of PER2 competes with phosphorylation to further regulate the circadian clock (Kaasik et al., 2013). In any case, the PER/CRY heterodimer is the key negative regulator of the canonical clock, and ultimately, the transcriptional activity of CLOCK/BMAL1 is altered if PER/CRY is destabilized, leading to a change in clock speed and/or phase. Because many of the aforementioned proteins have multiple binding partners, it is likely that they have secondary or tertiary effects on other physiological processes outside their primary roles in clock regulation (Grimaldi

et al., 2010). The molecular pathways regulating sleep homeostasis remain less well defined and present an exciting and challenging frontier of investigation. Indeed, many of these clock genes have associations with sleep regulation, providing an initial window into the connectivity between circadian processes and parameters of sleep, thus reinforcing the idea that sleep is perhaps gated by the circadian clock (Fisher et al., 2013; Mang & Franken, 2013).

Some heritable sleep conditions, such as familial advanced sleep phase (FASP) and familial natural short sleep (FNSS), were previously termed as disorders but have since been reclassified as traits because of the perceived advantage (by many) that they confer to carriers. We consider them “disorders” only in cases where affected individuals are troubled by the trait. Interestingly, genetic drift selects against populations of mice carrying the *tau* mutation in casein kinase 1 ϵ (which causes shortened period and accelerated free-running circadian cycles), when allowed to mate freely over time (Spoelstra, Wikelski, Daan, Loudon, & Hau, 2016). Yet, our uniquely human environment and social demands may be a reason why these traits exist in the larger population. Studies of twins leads to estimates of genetic contributions to sleep quality (33%) and to sleep pattern (as defined by bedtime, sleep time, sleep duration, and daytime napping) (40%) (Heath, Kendler, Eaves, & Martin, 1990) establishing precedence for studying the genetics contributing to a complex behavioral trait. By studying individuals with heritable sleep traits, we are able to clarify mechanistic details of sleep or circadian regulation and also to identify phenotypes that are stable at the human organismal level, thus providing an unprecedented approach to identifying putative therapies for sleep and circadian disorders affecting the larger population. The relative conservation of genes across species underlies the fundamental importance of these pathways in regulating the critical physiological process of circadian regulation and sleep. Because these pathways exist in humans, the confirmation, post-transcriptional regulation, and fine-tuning of these molecular elements have been significantly impacted by human genetics and in particular forward genetic screens using human subjects with heritable sleep traits (which will be discussed in detail in the following text) (Hsu, Ptáček, & Fu, 2015).

In this chapter, we will outline an approach to identifying specific causative mutations for circadian traits that alter the timing of sleep-wake behavior and characterizing these alleles in the laboratory. We will provide additional information from selected genetic association studies for single-nucleotide polymorphisms (SNPs) linked to circadian conditions. Alleles discovered using the same approaches that inform the regulation of homeostatic sleep drive will also be outlined briefly.

II METHODOLOGY

The main goal of research into the genetic basis of sleep and circadian traits is to identify specific variants in the genome that cause phenotypes and then to study the mechanisms by which these mutations lead to the sleep or circadian trait. Studying sleep behavior is complicated. Because sleep research is a relatively nascent field with its modern origins in the early 1970s, the classification of sleep disorders is still developing, both in terms of identified diseases/traits and refinement of phenotyping of such diseases/traits. Sleep and circadian traits are complex behaviors, and they are genetically and mechanistically diverse. Inherent genetic and biological tendencies are subject to external factors, which can be confounding. Some of these confounds include considerable variability in assessed phenotypes, overlapping psychological pathology, environmental factors like medication or other drugs, social and cultural influences, and school/work pressures, all of which make objective assessments of sleep in large populations difficult to obtain (Chong et al., 2015; Hsu et al., 2015; Veatch, Keenan, Gehrman, Malow, & Pack, 2017).

To achieve this goal, there are generally two genetic approaches that have been applied to study sleep behavior, and they differ largely in the subjects that are used as inputs for study. One approach, known as genome-wide association studies (GWAS), centers on an unbiased assessment of associations between specific phenotypes and gene variants across many thousands of genomes. The other, known as a forward genetic approach, focuses on very careful phenotyping of affected individuals within families to identify genetic variants by candidate gene sequencing and whole-exome or whole-genome sequencing that segregates with the affected family members. Once a putative allele is identified, modeling of this mutation is done *in vitro* and *in vivo* in animal models to determine whether the allele is sufficient to reproduce the phenotype, with the given caveats and limitations of behavioral or molecular differences in various model systems.

Genome-wide association studies: With access to the genomic data from thousands of individuals from accumulated sequencing projects, there is an increasing ability to correlate genetic variants to specific traits, in addition to providing a greater understanding of the genetic variations that exist in the human population. Genome-wide association studies (GWAS) are observational studies that seek associations between traits or diseases in a population of participants with specific genetic variants across their sequenced genomes. This approach is generally considered to be hypothesis-free (Gehrman, Keenan, Byrne, & Pack, 2015). If a variant segregates with a disease, then that region of the genome, allele, or even a

specific SNP may contain information that contributes to the development of that disease. GWAS studies focus on common genetic variants that reach statistical significance but typically have very small effect sizes on complex phenotypes, which certainly is applicable to the complex behaviors related to sleep regulation. Because of the stringent statistical thresholds required for GWAS and the expected small effect size, these studies require sequencing data from many thousands of individuals (Gehrman et al., 2015). While these studies have yielded some interesting findings, many associations have not been replicated, thus calling into question whether the association is real. Also, for every association reported, very few have been followed up to determine the genetic and molecular basis of the genetic variant causing increased risk for that phenotype. Another problem with GWAS is that such associations do not necessarily represent the causative variant, which may be in linkage disequilibrium with the phenotype in question. Further, even if a variant that contributes to increased risk of a disease is identified and resides in noncoding sequences, it is not necessarily given that it acts via the nearest gene. Some intronic and intragenic variants can act on gene regulation more remotely. Thus, without detailed functional work to define the relevant gene/pathway in which a risk allele may act, determining its precise contributions to pathophysiology remains largely speculative. In contrast, a forward genetic approach in families with Mendelian inheritance of a trait incorporates molecular and biochemical experimentation to demonstrate causality.

Forward genetic approaches: Because complex behavior has many potential confounding factors that make an analysis of causative alleles difficult, the second approach focuses on clinical diagnosis where these sleep and circadian behavioral variables are meticulously accounted for before elucidating a genetic cause. So far, this approach has been used successfully in characterizing familial advanced phase (FASP) and familial natural short sleep (FNSS) phenotypes.

FASP is a trait where affected individuals in a family wake up and sleep at much earlier times than unaffected members, while sleep quality and quantity are normal (Jones et al., 1999). FNSS is a trait where affected family members sleep significantly less than unaffected members but with normal circadian timing. The first step in studying the genetics of these traits is to identify affected and unaffected individuals in families through rigorous phenotyping. Careful attention must be paid to extreme, yet normal (nongenetic), variation in sleep onset and duration, adherence to established thresholds (defined to select for extreme phenotypes), and environmental or psychological influences that interfere with the phenotype of interest (Hsu et al., 2015). Subjects are recruited

either by referral from clinicians or by self-referral. Once identified, previous successful studies have demonstrated that selecting probands with relatively large deviations from the population mean can improve the effect size. The appropriate selection parameters for FASP are a sleep onset prior to 8:30 p.m. and spontaneous awakening before 5:30 a.m. and a sleep duration of 6.5 h or less for FNSS (Hsu et al., 2015; Jones, Huang, Ptáček, & Fu, 2012). Clinical evaluation of other health conditions and medications is imperative to determine their contributions to the phenotypes of interest. Also, special attention must be paid to environmental influences, such as exposure to light in the sleeping period, which is integrated into the patient evaluation. The use of established questionnaires and structured interviews is designed to distinguish innate sleep preferences from social and environmental obligations confounding sleep behavior. The Horne-Ostberg morning-eveningness (Horne & Östberg, 1976) and Munich Chronotype questionnaires (Roenneberg, Wirz-Justice, & Mellow, 2003) differentiate between the preference for a sleep pattern, as compared with the actual sleep patterns. Further, the Epworth and Karolinska sleepiness scales are valuable in assessing the sufficiency of sleep in a given individual (Åkerstedt & Gillberg, 1990; Hsu et al., 2015; Johns, 2002).

Following up on self-reported data first involves a structured interview that focuses on identifying environmental (psychosocial and familial-cultural) influences that might alter the biological (genetic) timing of sleep. Whenever possible, it is worthwhile to gather objective data to help characterize either advanced sleep phase (ASP) or natural short sleep (NSS) behaviors, including sleep logs, actigraphy, and measurement of melatonin profiles. Further, there are a variety of more labor-intensive approaches to analyze circadian cycles and sleep patterns in humans, including polysomnography in a sleep lab or (via telemetry) in the home environment.

The objective assessment of ASP usually involves an extended period in a constant environment with minimized exposure to environmental time cues (free running) (Kleitman & Kleitman, 1953) or by imposing the subject to 20–28 h of rest-activity periods (forced desynchrony) (Dijk, Duffy, Kiel, Shanahan, & Czeisler, 1999). These manipulations allow the assessment of endogenous circadian period, which, if shortened, has been shown to cause FASP (Hsu et al., 2015). Obviously, there are challenges with subjecting research subjects to these intensive and costly assays. Other analyses that focus on phase can follow surrogates of circadian rhythmicity to assess for physiological circadian peaks and nadirs relative to time cues in a noninvasive manner. The best example of this is the use of salivary melatonin levels to assess dim light melatonin onset (DLMO), since the circadian-derived peak of melatonin correlates with the onset of sleep timing (Lewy, 2007), which is expected to

shift forward in FASP subjects relative to unaffected controls (Jones et al., 1999). The use of actigraphic measures can also provide further insight into rest-activity patterns in the real world, especially when used in the general population, and may be a useful tool for future investigations of phase and short sleep phenotypes.

Objective assessment of NSS is centered on quantifying not only the duration of sleep but also the parameters that define the homeostatic sleep pressure (Process S) in both wake and sleep. To that effect, actigraphy may be useful to measure inactivity as a surrogate for the duration of sleep. However, even though actigraphy can be employed as an initial screening tool, electroencephalography (EEG) remains the gold standard for markers of sleep pressure. In particular, EEG data provide precise information about the onset and offset of sleep, the timing of various sleep stages, and the intensity and specific EEG frequency components of sleep. For example, slow-wave activity (frequency 0.5–4 Hz) is a marker of sleep depth in NREM epochs and sleep drive during the course of waking periods (Dijk, Beersma, & van den Hoofdakker, 1989). Responses to sleep deprivation can also be studied using the EEG, with NSS individuals presumably exhibiting reduced NREM and REM duration in normal sleep and reduced NREM and REM during the recovery period after sleep deprivation.

Once an individual is classified as a carrier of the trait, DNA samples are collected from as many informative individuals in the kindred as possible, including both affected and unaffected (at-risk) members of the family and both parents. The primary sampling method is to collect blood, where high-quality DNA can be extracted and analyzed using a variety of genetic sequencing methodologies. Using this forward genetic approach, we have focused on phenotypes that have an autosomal dominant pattern of inheritance. Because the incidence of FASP and FNSS are relatively low in the population, the implicated alleles are likely rare variants with a large effect size for the phenotype (Hsu et al., 2015). For large families with multiple affected family members, linkage analysis can be performed to rule out large parts of the genome. Linkage analysis takes advantage of the fact that SNPs (markers) that are in close proximity to causative alleles on chromosomes are more likely to be passed down to offspring (after meiosis) than genes that are farther away (Gehrman et al., 2015; Hsu et al., 2015). Thus, the locus harboring the disease variant can be identified by tracking and comparing the marker and phenotype between affected and unaffected family members, as was done in the original characterization of FASP. The maximum likelihood logarithm of odds (LOD) score is a statistical measure of the likelihood of linkage for each identified region. Higher LOD scores indicate a greater significance of linkage with the phenotype (vs the alternative hypothesis, i.e., that they are cosegregating based on

chance). A LOD score of ≥ 3 is considered statistically significant for linkage (Gehrman et al., 2015; Hsu et al., 2015). Once a statistically significant LOD score implicates a locus as being linked, fine mapping is performed to define the minimum critical region where a causative mutation must reside. Sequencing genes in this chromosomal region of interest can then identify the specific causative mutation.

A majority of identified FASP and FNSS kindreds are characterized based on definitive information from only a small number of family members (two to three affected individuals). In this case, it is not possible to definitively map the genomic locus. However, studies of cosegregation of candidate mutations with the phenotype are helpful in ruling out many variants (Hsu et al., 2015). To identify associated alleles in these families, a candidate gene (using *a priori* information from previous studies) approach has been very successful for FASP, but is not possible for FNSS, given the current limited understanding of the molecular regulators and pathways governing homeostatic sleep. Another powerful approach is whole-exome/whole-genome sequencing in groups of affected individuals, which allows the identification of a large number of variants that can then be sifted and prioritized to define a list of high-priority putative causative alleles for further investigation. In such studies, the strongest genetic data are provided by identifying multiple rare alleles in the same gene in DNA from unrelated individuals with the same phenotype. To add weight to causality of a particular allelic variant, consideration of sequence homology across phylogeny points to functional importance and, thus, provides strong support for a (causal) role of a specific variant. This approach has been successfully applied to small kindreds in prior studies (Hirano et al., 2016; Xu et al., 2005; Zhang et al., 2016) but should nevertheless be employed cautiously, as there is little statistical support for identified disease alleles (Hsu et al., 2015). Further characterization of these alleles in model systems (*in vitro* and *in vivo*) becomes essential to substantiate causality.

III HERITABLE SLEEP TRAITS

The most recent International Classification of Sleep Disorders (ICSD) lists roughly 60 disorders of sleep in humans (Sateia, 2014). A smaller subset of these conditions has been recognized to have a heritable component. Similar to the parsing of sleep into Processes C and S, these heritable conditions largely fall into either those that broadly affect circadian rhythmicity or sleep homeostasis/duration. While it is plausible that there are phenotypes that affect both Processes C and S, they have yet to be linked to novel alleles, or established variants have been insufficiently studied to identify the overlap.

Among the various classified sleep homeostasis/duration conditions, narcolepsy, restless leg syndrome, obstructive sleep apnea, fatal familial insomnia, natural short sleep (NSS), and REM sleep behavior disorder have varying levels of defined heritability. There are fewer recognized circadian traits, comprising six distinct entities: advanced sleep phase (ASP), delayed sleep phase (DSP), shift work disorder, jet lag disorder, free-running sleep (or non-24 hour sleep-wake rhythm) disorder, and irregular sleep-wake disorder, with ASP and DSP having genetic associations. In the remainder of the text, we will focus on the traits that are involved with the timing of sleep-wake behavior, with a specific focus on a detailed discussion of ASP. Further, we will outline the genetic variations contributing to the altered sleep homeostasis/duration in humans with a focus on NSS.

Morning or evening preferences (as assessed by Horne-Ostberg questionnaires to classify individuals as “typically” morning or evening types) are linked to SNPs, many of which are in known circadian clock genes. One polymorphism is in the *CLOCK* gene in the 3'-untranslated region of the transcript (Zhang, Ptáček, & Fu, 2013). Carriers show a delay in preferences for sleep and activity that ranges from 10 to 44 min compared with noncarriers (Benedetti et al., 2008; Garaulet et al., 2011; Mishima, Tozawa, Satoh, Saitoh, & Mishima, 2005). SNPs in *NPAS2* are associated with the self-reported timing of sleep and adaptability and sleepiness in nurses on shift work schedules; interestingly, both of these SNPs localize to intron three of the *NPAS2* gene (Gamble et al., 2011). A locus in exon 18 of the *PER1* gene is associated with extreme morning and evening preferences. The 2434 T/C allele is a silent variation, where the C in this position associates with extreme morning preference compared with subjects with extreme evening preferences (Carpen, Von Schantz, Smits, Skene, & Archer, 2006). *PER2* is linked to diurnal preference, with an SNP in the 5'-UTR-111G upstream of the start codon causing a predicted alteration of the mRNA secondary structure, leading to an association with extreme morning preferences (Carpen, Archer, Skene, Smits, & Von Schantz, 2005). A second missense variant in *PER2* (1244 Gly/Glu) is also associated with morningness in a Korean population (Lee et al., 2011). The consistent association of morning or evening preferences with genetic variants in identified circadian clock genes reinforces the role of the clock in the timing of sleep-wake behavior.

Delayed sleep phase (DSP) is characterized by sleeping and waking significantly later than the majority of the population. Phase advances and delays are considered “disorders” only when the trait is perceived negatively by the individual, which is more common with DSP than with ASP. Such cases lead to the diagnosis of either DSP disorder (DSPD) or ASP disorder (ASPD). Individuals with DSPD often have significant conflicts with expected

social and work requirements that adhere to schedules based on optimal timing for unaffected individuals. As a result, DSPD patients are often (incorrectly) diagnosed with insomnia, because of excessive daytime fatigue resulting from their attempts to adhere to “normal” schedules (Chong et al., 2015; Jones et al., 2012). Specifically, these individuals initiate sleep well after midnight and prefer to wake up in the late morning or afternoon; some cases of DSPD exhibit a delayed phase of melatonin rhythms to corroborate a shifted sleep-wake phase (Jones et al., 2012). This condition has been associated with mutations in aralkylamine N-acetyltransferase (AANAT, the rate-limiting enzyme functioning in the synthesis of melatonin) (Hohjoh et al., 2003) and the *PER3* variable number tandem repeat locus (discussed later), but further genetic evidence or confirmation in model systems is lacking (Archer, Schmidt, Vandewalle, & Dijk, 2018). A variant in *CRY1* (c.1657+3A>C) was recently identified to be a likely causal mutation for DSP based on a detailed phenotyping of a single proband and a control subject and subsequent candidate gene sequencing of the proband’s small kindred (Patke et al., 2017). This variant is situated at a position that causes splice site disruption and exon skipping, thereby producing an in-frame deletion of 24 amino acids in the C-terminal region of the protein. This deletion causes increased repressor activity of *CRY1* for the *BMAL/CLOCK* heterodimer *in vitro* and lengthens circadian period in cell culture systems. Because this allele has a frequency of up to 0.6% (based on available genomic databases), the authors were able to reverse phenotype 70 subjects (homozygotes, heterozygotes, and noncarriers) in six unrelated families. Of the 38 carriers of the *CRY1* mutant allele, most reported sleep disturbances inclusive of delayed sleep times and increased sleep fragmentation (Patke et al., 2017). Although there is *in vitro* evidence supporting the possible causal role of the variant, it remains to be demonstrated that the variant will produce similar phenotype in a mammalian model system such as mouse.

Familial advanced sleep phase (FASP): ASP is a condition where affected individuals have earlier sleep and wake times than unaffected individuals. The criteria for the clinical diagnosis of ASPD are based on a patient’s complaint of a sleep time that is earlier than his/her “desired” sleep schedule (Jones et al., 2012). We defined FASP in families segregating ASP as an autosomal dominant trait. Individuals with FASP experience pronounced difficulty staying awake in the evening and an obligate need to wake up significantly earlier than nonaffected individuals. Among affected individuals in a family, some are troubled by the trait, others are indifferent to it, while yet, others feel “virtuous” for being the “early bird that gets the worm.”

When first described, the proband presented to the clinic with “disabling early evening sleepiness and early morning awakening” (Jones et al., 1999) and the

recognition that other family members had similar traits lead to the classification of heritability (Jones et al., 1999; Toh et al., 2001). The phenotype is segregated in a highly penetrant autosomal dominant manner in this family. The typical onset of FASP is relatively early in life, with the youngest affected individual being 8 years old and most affected family members being aware of their early morning preferences by the age of 30. The proband had a dramatic advancement in sleep phase of 4–6 h compared with controls, subsequently affected family members also showed objective phase advance of nearly 4–6 h in sleep onset and offset, DLMO and temperature nadir compared with unaffected family members. Overall quality and quantity of sleep, as measured by polysomnography, were normal. One affected individual in the kindred was studied in a time isolation facility, and her endogenous circadian period, as measured by sleep-wake activity patterns and temperature data, was shortened to 23.3 h compared with 24.2 h in matched controls (Jones et al., 1999). Based on these data, FASP was defined as an autosomal dominant human sleep trait characterized by stable entrainment of sleep and wakefulness to early solar times (Jones et al., 2012).

To determine the genetic basis of FASP in this extensively phenotyped large kindred, linkage analysis was performed, which mapped the location to chromosome 2qter with a LOD score of 5.25. Further investigation into this region revealed approximately 40 cDNAs, all of which were sequenced, recognizing *PER2* as one of the candidates (Toh et al., 2001). Sequencing the *PER2* gene in the FASP kindred revealed four changes, three of which were at wobble positions, but the fourth base change at position 2106 (A–G) was predicted to cause an amino acid substitution from serine to glycine at position 662 in the *PER2* protein (S662G). This mutation segregated with the affected individuals in the family and was not found in control samples. To test the functional consequences of this mutation, *in vitro* experiments were performed using *PER2* truncation mutants and immunoprecipitation assays to show that the S662G mutation resides within a CK1-binding domain. Assaying gel-mobility shifts of wild-type (WT) or mutant *PER2* fragments incubated with CK1 revealed that the S662G mutation abolished phosphorylation in this region. Consistent with the CK1 recognition motif (S-X-X-S-), amino acid sequences of the residue downstream to S662 showed serines at positions 665, 668, 671, and 674. Mimicking phosphorylation at 662 by substituting the serine with phosphomimic aspartate (S662D), CK1-dependent phosphorylation was restored to the subsequent serines (665, 668, 671, and 674) suggesting CK1 is responsible for the phosphorylation of these subsequent serines. Additional studies confirmed this result by using *PER2* peptide fragments with a covalently linked phosphate at serine 662 (Xu et al., 2007). Separate investigations using cell

culture demonstrated that the PER2 S662G mutation results in reduced protein stability (Vanselow et al., 2006), suggesting alternative sites of phosphorylation that may target the protein for degradation. It was reported that CK1-mediated phosphorylation of PER at these alternative sites leads to the recruitment of specific ubiquitin ligases, causing ubiquitylation and degradation of PER (Eide et al., 2005; Luo et al., 2009; Reischl et al., 2007; Zhang et al., 2013). Furthermore, CK1 was recently identified as the kinase responsible for phosphorylating PER2 S662 (Fustin et al., 2018; Narasimamurthy et al., 2018). These data demonstrate that the PER2 S662G mutation causes a deficit in phosphorylation at a key priming site required for a downstream phosphorylation cascade by CK1 and alters its stability, possibly by unbalanced phosphorylation.

These studies unveiled important pathways for the regulation of PER2 *in vitro*, but modeling of this mutation in mouse models provided the necessary step in understanding FASP. Transgenic mouse models carrying the mutant human (h) PER2 S662G, S662D, and wild-type (WT) sequences with the appropriate regulatory domains were generated (Xu et al., 2007). Behavioral analysis of these animals demonstrated a recapitulation of the FASP phenotype. hPER2-S662G animals had a dramatically shortened period of 22 h in constant darkness (DD) and a 4–6 h phase advance of rest/activity rhythms in 12 h light/12 h dark (LD 12:12). The hPER2-S662D mice had period lengthening of 0.5 h in DD and did not display a change of phase in LD 12:12, and hPER2 WT mice had normal period of 23.7 h and normal phase. Analysis of PER2 protein from affected and control human fibroblasts showed reduced levels of PER2 protein in S662G mutants compared with WT but no change in the relative nuclear content, which was confirmed with the transgenic mouse lines. Time-course analysis of mRNA transcripts from transgenic mouse tissue showed that hPER2 and endogenous mouse (m) *Per2* peak earlier in S662G mice compared with WT, consistent with the ASP phenotype, and as expected, the S662D mice had later peaks. Both m*Per2* and hPER2 RNA levels were reduced in the S662G line, consistent with a stronger transcriptional repressor activity on CLOCK/BMAL1 by the mutant S662G PER (Kaasik et al., 2013; Xu et al., 2007).

In another smaller FASP kindred (K5231), a candidate gene sequencing approach for known circadian genes was employed to screen members of the family. An A-to-G change was identified in the DNA sequence of *CK1δ*, which results in an amino acid change at position 44 from a threonine to alanine in the protein (CK1δ T44A) (Xu et al., 2005). This mutation cosegregated with the phenotype in the family and was novel. Phylogenetic analysis of this residue showed conservation in humans, mouse, and *Drosophila*, suggesting its importance in kinase function across species. *In vitro* analysis of the

CK1δ T44A compared with WT showed that it has decreased kinase activity for the exogenous substrates phosvitin and α-casein and even further reduced phosphorylation of PER 1–3 substrates. Transgenic mice were created containing the appropriate cis-regulatory elements with either mutant hCK1δ-T44A or wild-type hCK1δ-WT genes to evaluate the effect of the mutation *in vivo*. In DD, period length was significantly shortened in the hCK1δ-T44A (23.4 h) versus hCK1δ-WT (23.8 h), and mCK1δ heterozygous (+/−) mice had a period of 23.7 h. Activity of behavior in LD 12:12 did not reveal a sleep phase advance. When reentraining to LD 12:12 from an extended period of DD, a deficit in time to reentrainment was observed in the hCK1δ-T44A mutant animals. Because CK1δ was known to regulate PER2 function in modulating clock speed and circadian timing, the effect of crossing hPER2-S662G, hPER2-S662D, and hPER2-WT to hCK1δ-WT or mCK1δ^{+/−} mice was examined (Xu et al., 2007; Zhang et al., 2013). Period length of the crosses derived from the mutant but not the WT hPER2 transgenic mice was affected. Crosses of hPER2-S662G and hCK1δ-WT shortened period by an additional hour (20.8 h) compared with hPER2-S662G (22.0 h). When hPER2-S662G was crossed to the mCK1δ^{+/−}, period was lengthened (22.3 h) compared with control hPER2-S662G animals. These experiments suggest that decreasing CK1δ dosage lengthens period for hPER2-S662G or, conversely, increasing CK1δ dosage shortens period of S662G mutants. This experiment further supports the notion that the PER2 S662G site is a critical residue for the downstream phosphorylation cascade by CK1δ. When this site is unable to be phosphorylated, subsequent phosphorylation of downstream sites is blocked, and other sites outside of this region are phosphorylated. This leads to targeting of PER2 for degradation, thus tipping the equilibrium toward reduced PER2 levels and causing shortened period. These data confirm that the posttranslational regulation of PER2 is sufficient to cause FASP and, importantly, have implications for the regulation of sleep timing in the human population more generally. Interestingly, the mutation carriers in this family all have migraine, and notably, an additional allele, *CSNK1D-H46R*, has also been identified to be associated with FASP and familial migraine. The role of *CSNK1D* in causing FASP with migraine was further supported by *in vitro* and *in vivo* experiments (Brennan et al., 2013).

Candidate gene screening was again applied to another small-sized FASP kindred (K50035), identifying a missense mutation in the human *CRY2* gene that converts an alanine to threonine at position 260 in the amino acid sequence (A260T) that cosegregates with affected individuals (Hirano et al., 2016). The proband's FASP phenotype was confirmed with melatonin sampling, with an onset that is advanced by >4 h (4:40 p.m. compared with 8:50 p.m. for unaffected individuals).

The A260T mutation site is a highly conserved residue in vertebrates and is in the FAD-binding domain of CRY2. *In vitro* analysis of this mutation revealed that the A260T mutation has reduced repressor activity on CLOCK-/BMAL1-mediated transcription and that the mutated protein was more unstable compared with WT (Hirano et al., 2016). FBXL3 promotes the degradation of CRY2 through its association with the FAD-binding pocket (Xing et al., 2013). Because the mutation is located in the FAD-binding region, it was postulated that it alters the CRY2-FBXL3 interaction and promotes the instability of hCRY2-A260T. By knocking down FBXL3 in cultured cells, hCRY2-A260T stability was restored. Because FAD stabilizes CRY2 by interfering with the CRY2-FBXL3 interaction (Hirano et al., 2016), the ability of FAD to protect CRY2 from FBXL3 was interrogated using a competitive binding assay. When FAD was added to the assay, free hCRY2-WT protein levels were released in a dose-dependent manner, while hCRY2-A260T protein remained bound more tightly to FBXL3. Structural modeling of this mutation confirmed changes in the binding pocket that likely renders hCRY2-A260T more accessible to FBXL3.

Transgenic mice carrying the mutation (hCRY2-A260T) or wild-type (hCRY2-WT) genes were generated to test whether the A260T mutation causes FASP. In behavioral analyses under LD 12:12, hCRY2-A260T mice exhibited an earlier peak of resting behavior and activity onset and offset compared with hCRY2-WT mice. Period length was assessed in DD, and hCRY2-A260T had a shortened period (23.5 h) compared with hCRY2-WT (23.7 h). Both the advanced phase and shortened period behaviors are consistent with previous reports of FASP (Xu et al., 2007). To summarize these findings, the hCRY2-A260T causes a conformational change in the FAD-binding pocket of CRY2, increasing its net interaction with FBXL3, thus promoting its degradation and leading to reduced repressor activity on the CLOCK/BMAL1 heterodimer. This causes period shortening and advanced phase in the mouse and human. Because FAD is a key component in multiple metabolic pathways, a second set of experiments examined whether FAD can alter the stability of CRY2 and, thus, affect circadian rhythms in mice (Hirano, Braas, Fu, & Ptáček, 2017). Indeed, these complimentary experiments showed that FAD stabilizes CRY2 and increases period length in a dose-dependent manner *in vitro*. *In vivo*, components of the FAD synthetic pathway oscillate in a circadian manner, and knockdown of riboflavin kinase (Rfk, biosynthetic enzyme of FAD) alters clock gene expression (largely reduces expression) in the liver. Since FAD is derived from riboflavin (vitamin B2), these data suggest a novel pathway (informed by the findings in the FASP work) in the dietary and metabolic regulation of circadian proteins in peripheral tissues.

A mutation in *PER3* was identified in another small FASP kindred (K7107) with higher Beck Depression Inventory and seasonality scores, suggesting an overlapping seasonal affective disorder (SAD) phenotype (Zhang et al., 2016). Members of this family had a phase advance of roughly 4 h. The candidate circadian gene sequencing approach was applied and identified two missense variants on the same allele: a C-to-G change and an A-to-G change in *PER3*, which causes a proline-to-alanine amino acid change at 415 and histidine-to-arginine change at 417 in the protein. These rare variants were found to be conserved in vertebrate species, cosegregated with affected family members, and not identified in 250 control subjects. *In vitro* analysis of this variant demonstrated that hPER3-P415A/H417R has reduced repressor activity for CLOCK-/BMAL1-mediated transcription and reduced stability in the nucleus and cytoplasm compared with hPER3-WT. Previous studies had shown that *PER3* affects the stability of *PER1* and *PER2*. Here, hPER3-WT was found to help stabilize *PER1* and *PER2*, and hPER3-P415A/H417R lost this stabilizing effect of *PER1* and *PER2*. Wild-type (hPER3-WT) and mutant (hPER3-P415A/H417R) mice were generated and subjected to behavioral testing. Under standard LD 12:12 and DD conditions, there was no observed behavioral difference in phase or period length. Because of the seasonal light and mood sensitivity in the FASP human subjects, the mice were subjected to constant light (LL) and 4 h light/20 h dark (LD 4:20) to simulate extreme seasonal conditions. In LL, the period of hPER3-P415A/H417R mice was significantly longer (25.6 h) than that of hPER3-WT mice (24.9 h). In the LD 4:20 condition, hPER3-P415A/H417R mice had a dramatically altered phase in activity onset and offset patterns compared with WT and displayed lower amplitude of activity rhythms in the wheel-running paradigm used to assay rest/activity behavior. Tail suspension and forced swim tests (used as test for depression-like behavior) showed increased immobile time in hPER3-P415A/H417R mice compared with hPER3-WT, suggesting depression-like behavior, which was rescued with the administration of the antidepressant imipramine. Measures of depression-like behavior worsened when the mice were subjected to a short photoperiod, consistent with the phenotype of humans with seasonal affective disorder (Zhang et al., 2016). These data provide further insight into a developing mechanistic link between circadian rhythm maintenance and mood stabilization (Vadnie & McClung, 2017).

Common features of the reported FASP mutations include mutations in the key negative regulatory limb of the molecular circadian clock, altered repressor activity of the canonical heterodimer CLOCK/BMAL1, and protein instability of the mutated proteins, leading to alterations in period and changes in behavioral phase. However, it is possible that screens for FASP could yield

information on mutations that do not affect period, but specifically affect entrainment to light or coupling to other proteins that affect processes regulating sleep timing downstream of the clock itself. An in-depth investigation of FASP families has yielded substantial insight into the regulation of the circadian clock, particularly in its posttranslational modifications. This knowledge offers an opportunity to develop therapies in the future to adjust clock speed or phase in humans or better track and understand an individual's phase to appropriately time the administration of certain sensitive medications like chemotherapy or vaccinations. An interesting opportunity lies with ASP that commonly affects the elderly; due to its telomeric location, *PER2* expression could be affected by epigenetic changes associated with the aging process, causing the increased prevalence of ASP. It is possible that, by modulating the phosphorylation of *PER2* and increasing its stability, one could mitigate against this condition in elderly populations.

Sleep homeostasis traits include extreme variations in an individual's requirement or need for sleep. A 54-nucleotide variable number tandem repeat in exon 18 of the *PER3* gene has been associated with changes in slow-wave sleep during normal conditions and in recovery sleep after sleep deprivation. Individuals who are homozygous for the 5-repeat allele (*PER3* 5/5) have increased slow-wave activity in NREM sleep and increased alpha activity in REM sleep during baseline sleep that persist after sleep deprivation compared with individuals who are homozygous for four-tandem repeats (*PER3* 4/4) (Viola et al., 2012). Performance on neurocognitive tests during and after sleep deprivation is worse in *PER3* 5/5 compared with *PER3* 4/4 individuals (Viola et al., 2007). This polymorphism is also associated with self-reported adaptation to shift work in nurses and sleepiness during and after work shifts (Gamble et al., 2011).

Familial natural short sleep (FNSS): While evaluating human subjects with FASP, some individuals were noted not only to wake extremely early but also to fall asleep much later than typical FASP individuals. Further characterization of these families led to the recognition of an autosomal dominant familial form of natural short sleep (FNSS). The characteristic trait is a lifelong requirement of daily sleep time of approximately 6 h or less (He et al., 2009; Jones et al., 2012). FNSS individuals do not report a need for additional sleep or complain of sleepiness and perform well on cognitive testing under normal conditions. Although mouse models of some circadian clock genes were found to exhibit altered sleep homeostasis (Mang & Franken, 2013), FNSS was postulated to be the first human mutation recognized (not by association) to cause a specific sleep homeostasis (Process S) phenotype, as total sleep time is reduced in these individuals with otherwise stable circadian rhythmicity (Process C).

Kindred 7430 is a small family with two affected individuals (He et al., 2009). Sleep onset was roughly 10 p.m. with offset around 4 a.m. without an obvious phase phenotype. Sequencing of DNA identified a mutation in the *DEC2* (a transcriptional repressor known to inhibit CLOCK-/BMAL1-mediated transcription) gene with a proline-to-arginine alteration at amino acid 384 (hDEC2 P384R) in the protein that segregated with the phenotype. Phylogenetic analysis revealed conservation in mammals in a highly conserved proline-rich region near the HDAC-binding domain of unclear function. *In vitro* assays determined that the mutation reduced *DEC2* transcriptional repression of CLOCK/BMAL1 (He et al., 2009). Transgenic mice carrying the *DEC2*-P384R mutation (hDEC2-P384R) and wild type (hDEC2-WT) were subjected to circadian behavioral analysis and demonstrated no difference in free-running period in the DD condition. However, the duration of activity was significantly increased in hDEC2-P384 animals by 1.2 h. The hDEC2-P384R mice and WT littermate controls were then subjected to EEG analysis of sleep/wake behavior. Mutant mice had a significantly longer period of wakefulness and corresponding reduction in NREM and REM sleep. Analysis of EEG delta power across light and dark periods showed a non-significant increase at all times in the hDEC2-P384R line compared with controls. After 6 h of sleep deprivation in the first 6 h of the light period, mutant mice showed less NREM delta power in the rest phase immediately following the sleep deprivation protocol and less subsequent NREM and REM rebound sleep compared with WT controls, suggesting a decreased effect of sleep deprivation on the mutant animals. These data collectively indicate that the hDEC2-P384R mutation, indeed, causes a short sleep phenotype and may play a role in an accelerated recovery after sleep deprivation.

A separate experiment attempted to validate the finding that *DEC2* variants are involved in sleep homeostasis. Two large cohorts of twins, 59 monozygotic twins and 41 dizygotic twins, were subjected to acute sleep deprivation for 38 h (Pellegrino et al., 2014). EEG was applied to objectively quantify sleep-wake behavior, and subjects performed a 10 min psychomotor vigilance reaction time test during the sleep deprivation period. A novel variant in *DEC2* 1086 C-to-T nucleotide change corresponding to a change from tyrosine to histidine at position 362 (Y362H) in the amino acid sequence was found in a dizygotic twin pair (one noncarrier and one carrier) that was associated with fewer performance lapses during sleep deprivation and less total recovery sleep time after sleep deprivation (Pellegrino et al., 2014). Examination of the repressive activity of *DEC2*-Y362H *in vitro* demonstrated reduced activity, consistent with the findings for *DEC2*-P384R. Another recent study examined *DEC2* repressor activity and its relationship with the orexin pathway in sleep homeostasis. The expression of orexin was upregulated in hDEC2-P384R mice in the

hypothalamus at the transition from wake to sleep. *In vitro* assays demonstrated that DEC2 inhibits E-box-mediated transcription of prepro-orexin and that the P384R mutation decreases DEC2 repressor activity that is mediated through its binding and interaction with the MyoD1/E12 heterodimer (which drives the expression of prepro-orexin). The formation of the DEC2/MyoD1/E12 complex was weakened by the P384R mutation leading to increased prepro-orexin at the transition from wake to sleep, suggesting DEC2 regulates sleep duration, in part, by modulating the orexin signaling pathway (Hirano et al., 2018).

FNSS is a newly recognized entity in sleep genetics. While the exact roles of sleep are largely unknown (i.e., likely important for toxin clearance, synaptic homeostasis, and metabolic rebalancing), FNSS individuals appear to be efficient sleepers who are able to execute the necessary functions of sleep in a shorter amount of time and exhibit a higher resilience to sleep deprivation.

IV CONCLUSIONS

Due to the complexity of sleep behavior and the significant influence of the environment on sleep traits, delineating the pathways of sleep regulation has been a challenging enterprise. Using a methodical approach to phenotyping involving the objective characterization of sleep and circadian behavior in families with heritable sleep traits has led to the identification of genetic variants and subsequent characterization of molecular pathways in animal models by taking advantage of the conserved nature of this indispensable process. Insights obtained from FASP have pointed to the importance of posttranslational modification of circadian proteins to change circadian period or phase and fine-tune circadian timing. Further, FNSS constitutes an exciting field of research, where the identification of the first molecular pathways that regulate sleep duration now opens new opportunities to better understand sleep homeostasis. Understanding the fundamentals behind the timing and efficiency of sleep provides a framework that will allow for improved characterization of individual circadian phase or sleep need, more informed timing of medication delivery to improve efficacy, and, more broadly, the opportunity for the development of new therapies to assist with the consequences of sleep and circadian disruption in humans.

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